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Effect of Temperature on Lateral Gene Transfer Efficiency of Multi-Antibiotics Resistant Bacterium, *Alcaligenes faecalis*

(Kesan Suhu ke atas Kecekapan Pemindahan Gen Lateral Multi-Antibiotik Rintangan Bakteria, *Alcaligenes faecalis*)

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ABSTRACT

Antibiotic resistant bacterial population is a great threat for human as well as for other domestic animals. Unscientific use of antibiotics in clinical sectors create a selective pressure on bacteria that make bacteria resistant to a wide range of drugs. The current finding demonstrated the isolation and characterization of multi antibiotics resistant bacterial species from oil contaminated soil and its lateral gene transfer efficiency at different temperature. Among six bacterial isolates, the strain OD1 showed highest antibiotic resistant capacity was selected for further studies. Morphological, biochemical and 16S rDNA sequence analysis confirmed the bacterial strains as Alcaligenes faecalis *strain OD1. It was recorded that antibiotics resistant gene transfer was highly dependent on temperature and showed maximum transfer efficiency at 25°C (9.6 ×10^{-6}).*

Keywords: Antibiotics resistant bacterium; lateral gene transfer efficiency; temperature; 16S rDNA

ABSTRAK

Populasi antibiotik tahan bakteria adalah ancaman besar bagi manusia dan haiwan domestik lain. Penggunaan antibiotik tidak saintifik dalam sektor klinikal mewujudkan tekanan terpilih pada bakteria yang membuat bakteria tahan kepada pelbagai ubat-ubatan. Penemuan terkini menunjukkan pengasingan dan pencirian pelbagai spesies antibiotik tahan bakteria daripada minyak tanah tercemar dan kecekapan pemindahan gen lateral pada suhu yang berbeza. Antara enam pengasingan bakteria, terikan OD1 menunjukkan kapasiti antibiotik tahan yang tertinggi telah dipilih untuk melanjutkan pelajaran. Analisis morfologi, biokimia dan jujukan 16S rDNA mengesahkan terikan bakteria sebagai ketegangan Alcaligenes faecalis *OD1. Ia telah dicatatkan bahawa pemindahan gen antibiotik tahan terlalu bergantung kepada suhu dan menunjukkan kecekapan pemindahan maksimum pada 25°C (9.6 × 10-6).*

Kata kunci: Antibiotik rintangan bakteria; kecekapan pemindahan gen lateral; suhu; 16S rDNA

INTRODUCTION

In recent year, antibiotics resistant bacterial population has increased rapidly which is a major environmental concern. Random use of antibiotics in different sector includingin human health care units creates a selection pressure for antibiotics resistant bacteria (White et al. 2001). Uncontrolled use of antibiotics in different sector like fish farm (Son et al. 1997), swine farm (Gambarotto et al. 2001), poultry farm (Sorensen et al. 2001) and cattle farm (Snell 2008) creates a selection pressure for these drug resistant bacterial species. According to World Health Organization, these multi-drug resistant bacteria are rapidly spreading worldwide (WHO 2000). Centre of Disease Control (2010) reported that infection near about 30,000 people died in US in each year and its annual cost is about $1.87 billion (Aminov 2010). Antibiotics resistant property in bacteria may be innate or acquired. In this direction Giedraitienė et al. (2011) stated that bacteria aquire resistant properties in two ways, i. mutation in chromosomal gene and ii. gene transfer form one bacterium to another through plasmid. Researchers reported different types of multidrug resistant bacteria from different sources such as *Aeromonas* sp. from clinical area (Vilaa et al. 2002), *Enterococcus* and *Staphycoccus* from cattle farm (Chapin et al. 2004), *Salmonella* sp. from retail ground meat (White et al. 2001), *Pseudomonas aeruginosa* from waste water (Pandey et al. 2011). Higher level of antibiotics resistant property is found in those bacteria which are isolated mainly from health care unit and waste water (Aravena-Román et al. 2012; Pandey et al. 2011). Saiman et al. (2001) have reported multidrug resistant *Alcaligenes xylosoxidans* isolated from patient suffering from cystic fibrosis. Whereas, Lee et al. (2006) have reported the Postoperative endophthalmitis problem caused by *Alcaligenes faecalis*.

Lateral or horizontal gene transfer is the process of exchanging genetic material between two bacteria, which may be interspecies or intra-species. Prokaryotes, mainly bacteria are the most studied organism for lateral gene transfer (LGT) experiment. Gene transfer in bacteria occurs in response to changing environment which increase the genetic diversity and compatibility (Jain et al. 2003). Drug resistant property of the bacterial cells are due to presence of R plasmid which is considered as extra-chromosomal genetic material. Hancock (1997) has

reported that drug resistant property is much stronger in Gram negative bacteria compared to Gram positive bacteria due to presence of extra layer in cell wall. Researchers also have reported that *in vitro* conjugal transfer of different antibiotics resistant gene from one bacterial species to another (De & Deodhar 1995; Gevers et al. 2003). The transferable multidrug resistant property is a serious problem in the treatment against infection in human, as well as in other domestic animals. Though, several researchers have explored the phenomenon of bacterial gene transfer, but determinant factors during gene transfer/ conjugation are not clearly known. Bacterial conjugation is highly dependent on climatic conditions like temperature, pH, nutrient availability and salt concentration. Most of the studies related to bacterial gene transfer have been focused on medium pH (Palmen et al. 1993; Lorenz & Wackernagel 1994) and salt concentration (Page & Sadoff 1976; Page & Grant 1987). There are scanty of reports regarding the role of temperature on bacterial gene transfer. Therefore, the aim of our present investigation was to evaluate the importance of temperature on lateral gene transfer efficiency exhibited by the bacterial strain *Alcaligenes faecalis* (Acc No. KJ879243) isolated from oil contaminated soil.

Materials and Methods

ISOLATION OF ANTIBIOTICS RESISTANT BACTERIAL STRAIN

For this purpose, soil samples were collected from different petrol pump in Noida, Up, India. 1 g soil sample was dissolved in 10 mL 0.9% NaCl solution, serially diluted upto 10^{-10} and bacteria were isolated on tryptone soya agar plates (pH7.0) containing amphicillin at a concentration of 50 µg/mL. Bacterial colonies obtained after 24 h incubation period were pure cultured using repeated streaking method.

ANTIBIOTICS RESISTANT PROFILE OF THE SELECTED BACTERIAL STRAIN

Antibiotics resistant property of the selected bacterial strain was determined on tryptone soya agar plates (TSA) using HiMedia antibiotic kits. Three antibiotic kits were used, HX001 (Penicillin G, Oxacillin, Cephalothin, Clindamycin, Erythromycin, Amoxyclav), OD006 (Cephotaxime, Cephalexin, Co-Trimoxazole, Chloramphenicol, Nalidixic acid, Furazolidone, Norploxacin, Oxytetracycline) and HX022 (Amphicillin, Chloramphenicol, Penicillin G, Streptomycin, Sulphatriad, Tetracycline).

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

Minimum inhibitory concentrations (MIC) of the selected bacterial strain was checked in Mueller-Hinton agar plates (HiMediapvt. Ltd.) by disc diffusion assay method. 100 µL of 14 h culture of isolated bacterial strain was spreaded on plate and 5 mm pores were made by cork brudder. In each pore 30 µL of antibiotic solution at different concentration ranging from 10 mg/mL to 10 µg/mL was added and again incubated for 24 h at 37°C. Transparent zone around the well indicates sensitivity of the antibiotics. In this present study, five antibiotics, named amoxicillin, penicillin-G, levofloxacin, tetracycline and streptomycin were used at different concentration, 250, 100, 50, 25, 5 and 2.5 µg/mL.

CHARACTERIZATION OF THE SELECTED BACTERIAL ISOLATE

Characterization of the selected bacterial strain was primarily done by morphologically (Colony colour, elevation, surface, margin and Grams stain) and biochemically (citrate, esculin, nitrate, ONPG, methyl red, Voges-Proskauer, indole, H_2S, catalase, oxidase, gelatin, urease, glucose, fructose, starch, sucrose and lactose).

16S rDNA SEQUENCE ANALYSIS

The amplification of the 16S rDNA gene was done using forward primer 5′AGAGT TTGATCMTGGCTCAG 3′ (27F) and reverse primer 5′GGTTACCTTGTTACGACTT 3′ (1492R) at standard condition in Epindrof Thermal Cycler. Sequence data was edited, aligned, analyzed using Mega 4.0 and submitted to NCBI.

LATERAL GENE TRANSFER EFFICIENCY

Lateral gene transfer or conjugal efficiency was determined according to method of Snell (2008) with some modification. Due to lots of morphological difference from the donor strain *Alcaligenesfaecalis*, *Bacillus subtilis* (Acc. No. KF317211) was used as a recipient strain of R plasmid. 14 h culture of both donor and recipient cells were taken and diluted 10 fold upto 10^{-8} dilution. 100 µL of each diluted sample was then spreaded on TSA agar plate, incubated at 37°C for overnight and colony count was taken. For conjugation study, 1 mL of donor cell and 2 mL of recipient cells were mixed in 5 mL of TSA broth and incubated at different temperature varies from 20°C to 40°C for overnight. 100 µL of mixed culture was taken and diluted up to 10^{-10} dilution. 100 µL of diluted sample was spreadedon TSA agar plate containing 25 µg/ mL tetracycline and incubated at 37°C for overnight. The transconjugant colonies (recipient strain with antibiotics resistant gene) were counted and confirmed by Grams staining and antibiotics sensitivity test.

Results and Discussion

In this present investigation, multidrug resistant bacterial strains were isolated from oil contaminated soil from different petrol pump in Noida, UP, India. Among six strains, only one strain OD1 was selected for this present study due to its high antibiotics resistant property. Primarily antibiotic resistant capacity of the selected strain OD1 was checked using HiMedia antibiotic kits (Figure 1). It was detected that bacterial strain OD1 was resistant to most of the antibiotics, but sensitive to erythromycin

(Figure 1(a)) and Streptomycin (Figure 1(b)). The selected bacterial strain OD1 was found to be resistant against nalidixic acid also (Figure 1(c)). Primarily, the antibiotics resistant property was checked on HiMedia kit and tabulated in Table 1. Minimum inhibitory concentration of the selected bacterial strain was determined against six broad spectrum antibiotics (Table 2). Amoxycillin and tetracycline tolerant level was detected to be 50 µg/mL. Where as, penicillin-G and chloramphenicol were effective at a concentration of 250 µg/mL. The inhibition zone of the selected bacterial strain OD1 against these six antibiotics at different concentration was given in Figure 2. Levofloxacin (Figure 2(b)) and ciprofloxacin (Figure 2(e)) were detected to be most sensitive. The bacterial strain OD1 was primarily characterized by colony morphology and biochemical properties (Table 3). The bacterial isolate OD1 can actively utilized citrate and esculin, but negative in nitrate reduction. It showed positive reaction for catalase, oxidase, β-galactosidase, but negative in urease, methyl red and indole production. Carbohydrate utilization ability of the selected bacterial strain OD1 showed that it was unable to utilize glucose, fructose and starch. Finally the bacterial strain OD1 was identified as *Alcaligenesfaecalis* (Acc No. KJ879243) by 16S rDNA sequenceanalysis. The phylogenetic relationship with other close homologous strains was given in Figure 3. *Alcaligenes faecalis* is a citrate positive, urease and oxidase negative Gram negative bacilli which is considered as opportunistic pathogen. Saif et al. (1980) have reported the respiratory disease in commercial flocks in turkeys caused by *Alcaligenes faecalis*.

Lateral gene transfer (LGT) is the mechanism by which one bacterial species forward their plasmid to another bacterial species.The lateral gene transfer efficiency of

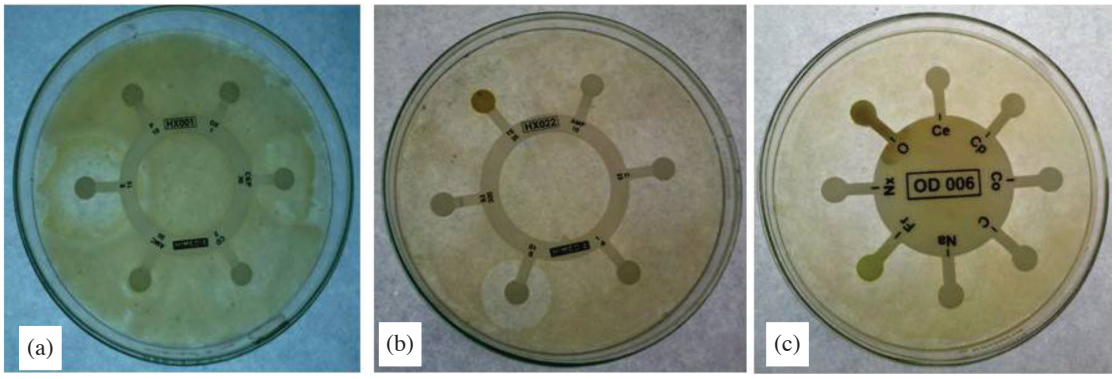

FIGURE 1. Effect of different antibiotics on the bacterial strain OD1. a) HiMedia kit HX001, b) HiMedia Kit HX022 and c) HiMedia Kit OD006

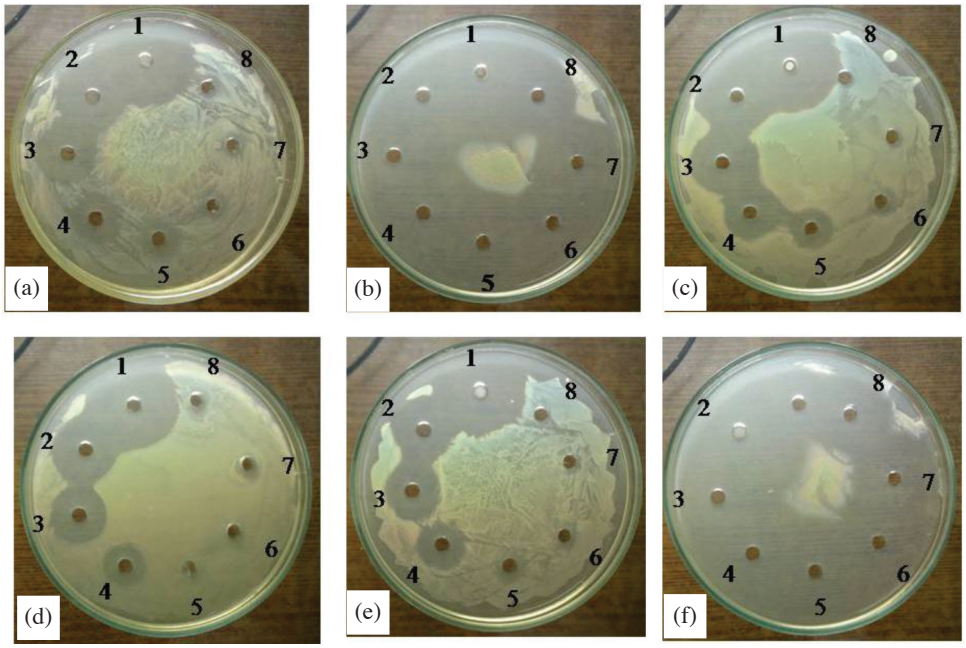

FIGURE 2. Inhibition zone against six antibiotics at different concentration. 1-8 represent 10, 1, 500, 250, 100, 50, 25 and 10 µg/mL, respectively. (a) Tetracycline, (b) Livofloxacin, (c) Amoxycillin, (d) Chloramphenicol, (e) Penicillin G and (f) Ciprofloxacin

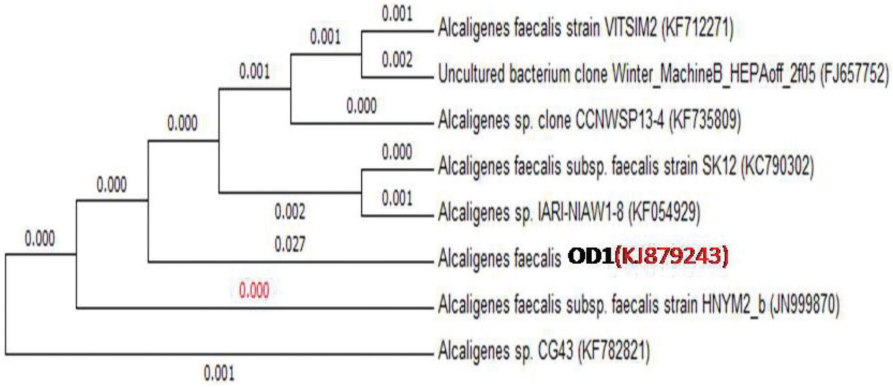

FIGURE 3. Phylogenetic relationship of the Bacterial strain *Alcaligenes faecalis* OD1 with other close homologous strains

TABLE 1. Determination of antibiotic resistant property on HiMedia kits

Antibiotics used	Concentration (mcg)	Bacterial strain OD1
HX001	5.98	R
Penicillin G	1	R
Oxacillin	30	R
Cephalothin	2	R
Clindamycin	15	S
Erythromycin	30	R
Amoxyclav		
HX022		
Amphicillin	10	R
Chloramphenicol	25	R
Penicillin G	0.59	R
Streptomycin	10	S
Sulphatriad	300	R
Tetracycline	25	R
OD006		
Cephotaxime	30	R
Cephalexin	30	R
Co-Trimoxazole	25	R
Chloramphenicol	30	R
Nalidixic acid	30	R
Furazolidone	50	R
Norploxacin	10	R
Oxytetracycline	30	R

R- resistant; S-sensitive; mcg-microgram

TABLE 3. Morphological characterization of the selected bacterial strain

Characteristics	Bacterial strain OD1
Colony morphology	
Pigment	Brown
Surface	Smooth
Margin	Irregular
Elevation	convex
Gram stain	Gram negative rod
Biochemical test	
Citrate	+
Esculin	+
Nitrate	-
ONPG	+
Methyl red	-
Indole	-
H_2S	-
Catalase	+
Oxidase	+
Glucose	-
Fructose	-
Sucrose	-
Starch	-

+ Positive reaction; - Negative reaction

TABLE 2. Calculation of minimum inhibitory concentration against six commonly used antibiotics

Concentration used	Amoxicillin	Penicilin-G	Tetracyclin	Levofloxacin	Ciprofloxacin	Chloramphenicol
10 µg/mL	R	R	R	S	S	R
25 µg/mL	R	R	R	S	S	R
50 µg/mL	R	R	R	S	S	R
100 µg/mL	S	R	S	S	S	R
250 µg/mL	S	S	S	S	S	S

R- resistant; S-sensitive

TABLE 4. Effect of temperature on conjugal gene transfer efficiency

No. of Donor cell in 1 mL	No. of recipient cell in 1 mL	Donor: recipient	No. of transconjugants in 1 mL at different temp.	Conjugation efficiency*
1.89×10^{11}	0.78×10^{11}	1:2	20°C: 1.11×10^{4}	5.8×10^{-8}
			25°C: 1.83×10^{6}	9.6×10^{-6}
			30°C: 1.52×10^{6}	8.04×10^{-6}
			35°C: 1.47×10^{5}	7.77×10^{-7}
			40°C: 1.92×10^{4}	1.01×10^{-7}

*Conjugation efficiency means number of transconjugants per donor cell

TABLE 5. Determination of minimum inhibitory concentration ofrecipient strain

Concentration Used	Recipient strain before gene transfer			Recipient strain after gene transfer		
	Amoxicillin	Tetracyclin	Chloramphenicol	Amoxicillin	Tetracyclin	Chloramphenicol
10 µg/mL	R	S	R	R	R	R
25 µg/mL	S	S	S	R	R	R
50 µg/mL	S	S	S	R	R	R
100 µg/mL	S	S	S	S	R	S
250 µg/mL	S	S	S	S	S	S

R- resistant; S-sensitive

the bacterial strain *Alcaligenes faecalis* OD1 at different temperature was tabulated in Table 4. It was detected that number of transconjugants (1.83×10^{6}) were higher at temperature 25°C i.e. lateral gene transfer efficiency was maximum at this temperature. Lateral gene transfer was highest at 25°C (9.6×10^{-6}), followed by 30°C (8.04×10^{-6}), 35°C (7.77×10^{-7}) and 40°C (1.01×10^{-7}). This investigation clearly depicted that with increase of temperature, gene transfer efficiency decreased gradually. To confirm the antibiotic gene transfer in recipient strain (*Bacillus subtilis*), antibiotic sensitivity test was again performed and presented in Table 5. It was detected that the bacterial isolate *Bacillus subtilis* become more resistant again antibiotics after gene transfer experiment. Previously, Singleton and Anson (1981) showed that conjugal transfer of R plasmid was dependent on temperature. The results of their study have also demonstrated that R plasmid transfer was maximum at temperature below 22°C. In our experiment, it was also recorded that LGT was maximum at 25°C. The decrease of the number of the transconjugants with increase of temperature proved the role of temperature in conjugation. In an investigation, Jain et al. (2003) have reported the influence of several internal (G/C content, genome size and nutrients) and external factors (temperature, salinity, oxygen & pressure) on LGT in prokaryotes. Whereas, Lorenz and Wackernagel (1992) has reported the efficiency of gene transfer in natural condition in various bacterial strains isolated from terrestrial and aquatic environment. Previously, Page and Sadoff (1976) investigated the LGT in *Azotobacter vinelandii* and recorded the optimum temperature as 30°C. On the other hand *Pseudomonas stutzeri* showed maximum gene transformation frequency at 20°C to 30°C (Lorenz & Wackernagel 1992). Not only temperature, there were others important factors that regulates the bacterial conjugation. Camacho and Casadesús (2002) have reported the role of leucine responsive regulatory protein and adenine methylation in conjugal transfer of R plasmid of *Salmonella enteric*. Bacterial competence development or uptake of foreign DNA is a popular and interesting topic to microbiologist. Though, several researches have been conducted to explore the mechanism of gene transfer in environmental conditions, but limited investigation was focused on temperature dependent gene transfer efficiency in bacteria.

CONCLUSION

Drug resistant bacterial population increase due to excess use of antibiotics in different sector and it creates a serious problem for animal health. Spreading of antibiotics resistant property to non- resistant bacterial strains through conjugation is the another big threat for environment. It is a great challenge for microbiologist to control these drug resistant bacterial species. This present investigation has confirmed the importance of temperature during conjugation process in bacteria. The investigated bacterial isolate exhibited an optimum LGT at 25°C and thus the country regions having average temperature between 20°C to 30°C will provide an ideal condition for emerging such multi drug resistant bacteria. Though bacteria grow optimally at 37°C, but higher temperature reduce the gene transfer efficiency. Particularly, during growth bacteria produce several types of secondary metabolites which might have some inhibitory activity in LGT process. Till date, no information is available in this particular topic. Further investigation should be conducted to explore the temperature dependent gene transfer mechanism in detail.

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